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14. ABSTRACT

We will determine the mechanism by which Shh signaling accelerates prostate tumor growth, identify Shh targets in prostate tumor stroma, and test the effect of individual target genes on tumor growth. The purpose of the report is to evaluate the second year of research. We reduced autocrine signaling in LNShh cells and found that this does not disrupt increased growth of LNShh tumors. This shows that autocrine Shh signaling is not involved in tumor growth. We evaluated Shh signaling in human prostate tumors. In our mouse model system, we have identified 9 stromal genes that may be involved in tumor growth that is stimulated by ShhOnly one gene is over-expressed in prostate cancer compared to normal prostate. Since Shh signaling is present in tumor stromal cells, we evaluated the role of different stromal phenotype in Shh signaling. Human benign and cancer samples were stained for myofibroblasts. We found that increased numbers of myofibroblasts relates to expression of each of the nine genes we identified in the xenograft model. Tumors with reactive stroma also have increased proliferation. These data may assist in predicting prostate tumor recurrence.

15. SUBJECT TERMS
tumor microenvironment, tumor-stromal interactions, Sonic Hedgehog, xenograft, reactive stroma

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INTRODUCTION

Background: Prostate cancer is the most prevalent cancer in men after skin cancer. The advent of PSA testing has led to a surge in the number of prostate cancer cases detected, but most men diagnosed with prostate cancer do not die of prostate cancer. One of the primary goals of prostate research today is to understand what makes some prostate tumors grow, while others remain indolent for decades. Prostate cancer growth is directed by stromal-epithelial interactions within the tumor. A stromal response is required for progression of prostate tumors and a normal stroma can revert the phenotype of a tumor to a less aggressive state. For these reasons, we suspect that alterations in prostate stroma are responsible for growth of aggressive tumors.

Many cancers arise from aberrant activation of developmental signaling pathways. Sonic hedgehog (Shh) directs stromal-epithelial interactions during embryonic prostate development. After budding initiates, Shh localizes to epithelial regions where prostatic buds appear. Shh signaling occurs in mesenchymal cells adjacent to the Shh expressing epithelium. Shh signaling in mesenchyme is presumed to induce epithelial proliferation, but the mechanism is unknown.
The Hedgehog signaling pathway has been implicated in prostate cancer. Shh ligand is expressed in adult prostate and signaling becomes activated in advanced prostate cancer. Shh overexpression increases the growth rate of tumors via an effect on the stroma.

Hypothesis: This proposal will address the hypothesis that Hedgehog regulated stromal target genes stimulate tumor growth.

Specific Aims: We will (1) determine the mechanism by which Shh signaling accelerates tumor growth, (2) identify Shh targets in prostate tumor stroma, and (3) test the effect of individual target genes on tumor growth.

Study Design: We will use our bi-clonal xenograft model to characterize the nature of Shh-induced signaling in LNCaP xenograft tumors in vitro and in vivo. We will determine if Shh-induced accelerated tumor growth is due to the action of a soluble, secreted factor, if the growth acceleration can be achieved by ligand-independent activation of Shh signaling in tumor stroma, and if the signaling components Gli1 and Gli2 are necessary for accelerated tumor growth. Then we will use the list of Shh target genes obtained previously by microarray analysis to identify Shh target genes that are induced in the stroma of LNCaP xenograft tumors, and also

present in human prostate tumors. Finally, the contribution of each of these target genes to Shh-induced tumor growth will be evaluated by gain-of-function and loss-of-function studies using our bi-clonal xenograft model.

Relevance: The strength of this proposal is that it is the first proposed model to identify stromal genes that regulate prostate tumor growth. We have known for decades that prostate tumor growth is dependent upon stromal influences, but we have as yet been unable to identify these influences due to the lack of a suitable model system. The stromal gene products identified in this study will provide a new class of therapeutic targets for preventing or slowing prostate cancer progression.

BODY

Task 1. **Determine the mechanism by which Shh signaling accelerates tumor growth. (Months 1-12)**

a. Determine whether paracrine effect is mediated by a soluble factor(s) (Months 1-6)

In vitro: LNCaP/LNShh were co-cultured with UGSM-2 cells and pulse labeled with BrdU to examine growth acceleration in LNCaP/LNShh cells. Shh does not accelerate growth of LNShh cells when they are co-cultured with UGSM-2 prostate stromal cells.

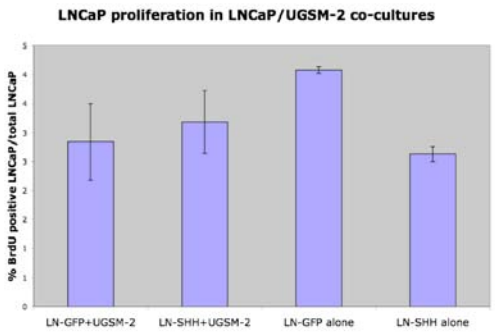


Figure 1. LNCaP/LNShh were plated on top of UGSM-2 monolayers for 48 hours, and then treated with BrdU for 30 minutes. Cells were immunocytochemically labeled for BrdU and analyzed using flow cytometry. The number of BrdU+ cells divided by total number of LNCaP is illustrated. Student's t-test revealed no significant differences.

We have provided many different models for this crucial interaction to occur. We provided different numbers of cells for different periods of time and in different culture medium. We included 3D co-cultures by culturing the cells on or within Matrigel or collagen gels. We analyzed LNCaP proliferation on cultures separated by porous membranes. We assayed LNCaP proliferation using methods other than BrdU incorporation. We analyzed LNCaP proliferation when treated with Shh treated UGSM-2 conditioned media. DHT alters the growth response of LNCaP and we

altered the DHT concentration in our co-cultures. Under all of these conditions, Shh does not induce proliferation of LNCaP cells.
This data is significant because stromal cells respond to Shh, but LNCaP do not have a growth response to stromal products. There are many reasons for this. There may be many paracrine products that alter the growth response of LNCaP and these products may come from several cell types. We may not be able to co-culture LNCaP and UGSM-2 cells long enough to recognize the growth response (cultures: up to 1 week, in vivo: 2-3 months). UGSM-2 cells may not provide the correct or the only paracrine response to Shh to induce LNCaP proliferation. Other stromal cells may contribute to the Shh growth response; i.e. endothelial cells, immune cells.
This model shows that culture conditions do not re-model tumor conditions that are required for Shh tumor growth. Therefore, co-culture model tasks for this research proposal must be limited to using xenograft or other in vivo techniques.
<u>In vivo</u>: We examined LNCaP proliferation in tumors composed of a 1:1 ratio of LN-Shh and parent LNCaP cells. We found that LNCaP proliferate faster than LNShh cells when included in the same tumor.

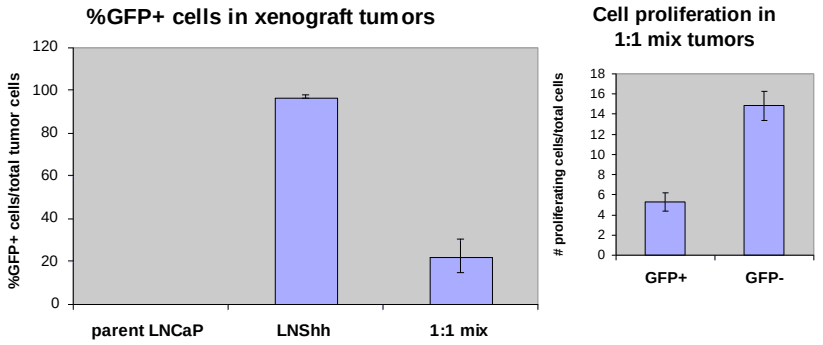


Figure 2. Equal numbers of LNCaP (GFP-) were mixed with LNShh (GFP+) cells and tumors were grown for 8 weeks. Proliferation of LNCaP/LNShh each was evaluated using GFP + Ki67 co-immunohistochemistry.

This identifies that Shh induces growth effects in LNCaP and LNShh cells in the same tumor. There are 2 options for this to occur: (1) LNCaP acquire a non-Shh dependent growth stimulus from LNShh cells (since neither LNShh nor LNCaP achieve Shh signaling in vitro), or (2) a secreted stromal factor must mediate the effect. Data to support option 2 is included in part 1b to conclude that stromal cells provide the Shh growth effect.
To examine the presence of autocrine Hedgehog signals in LNShh cells, we altered Gli2 function specifically in LNShh cells. Gli2 is the primary intracellular mediator of Hedgehog signaling (Bai 2002) and Gli2 activity can be reduced by expression of a dominant negative Gli2 mutant, mutB (Roessler 2005). We stably over-expressed mutB in LNShh cells. Since LNCaP do not respond to Shh ligand, we transiently expressed Gli2 in the cells and examined Hedgehog signaling by measuring Gli1. This showed

that mutB diminishes Gli1 transcription by Gli2. LNShh-mutB cells do not show any difference in proliferation in culture and LNShh-mutB xenografts grow at the same rate as LNShh xenografts. This shows that deletion of Gli2 activity in LNShh cells does not prevent enhanced growth of LNCaP tumors when Shh is over-expressed and argues against any autocrine growth effect.

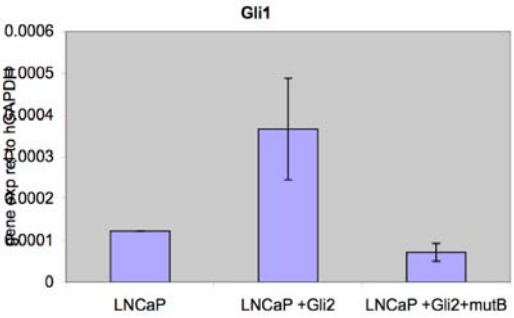


Figure 3. LNCaP were transiently transfected with Gli2, mutB DNA or both. Gli1 mRNA was analyzed by real-time RT-PCR. Gli2 increases Gli1 mRNA. Co-expression of mutB with Gli2 decreases Gli2 induction of Gli1 transcription.

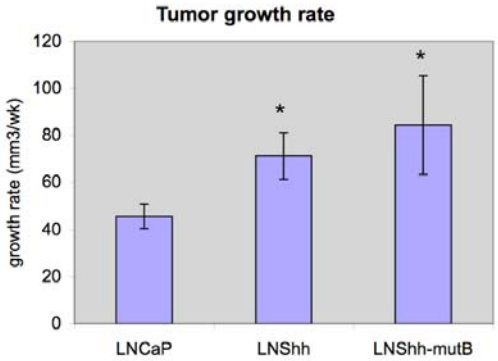


Figure 4. Xenograft tumor s were injected by injection of LNCaP, LNShh or LNShh-mutB cells. Tumors were measured weekly and tumor growth rates were calculated by determining the slope of growth of individual tumors and averaged. *indicates a significant growth rate increase over LNCaP xenografts.

b. Demonstrate that growth acceleration can be achieved by stromal cell SmoM2, Gli1 or Gli2 OE. (Months 1-12)

In vitro: will not be done (see 1a, in vitro)
In vivo: We overexpressed SmoM2, Gli1 and Gli2 in UGSM-2 cells. These cells showed increased transcription of Shh-induced gene products Gli1 and Ptc1. In culture, UGSM2-SmoM2 and UGSM2-Gli1 showed normal phenotype, but UGSM2-Gli2 showed an increased intensity to form anchorage independent structures. Anchorage-independence suggests that these cells may be tumorigenic.

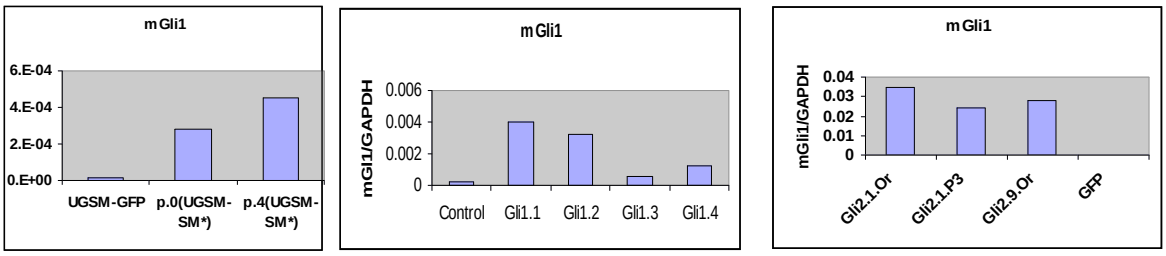


Figure 5. UGSM-2 cells were treated with human SmoM2 (left), Gli1 (middle) or Gli2 (right) retrovirus. Shh signaling in different passages of cells was determined by examining mouse Gli1 expression by RT-PCR.

LNCaP were co-injected with UGSM2-WT, UGSM2-Gli1 or UGSM2-Gli2 cells into nude mice and examined tumor growth rates. LNCaP + UGSM2-Gli1 tumors did not show any increased growth rate. LNCaP + UGSM2-Gli2 tumors showed a strong increase in growth rates, but this is attributed to UGSM2-Gli2 cells ability to form sarcomas (stromal-based tumors).

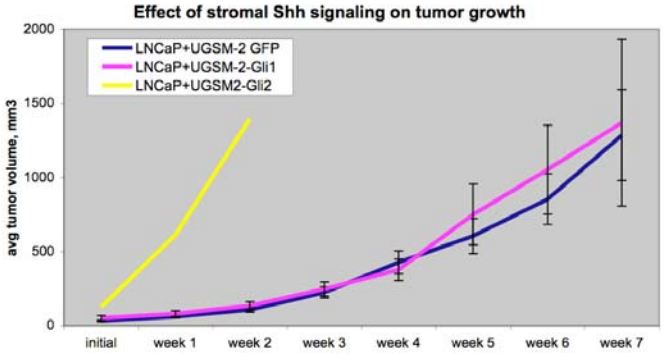


Figure 6. LNCaP were co-injected with UGSM2-SmoM2.Gli1/Gli2 cells. Tumor growth was measured as is illustrated as the average for each tumor type. LNCaP + UGSM2-Gli2 tumors formed rapidly and mice had to be sacrificed early.

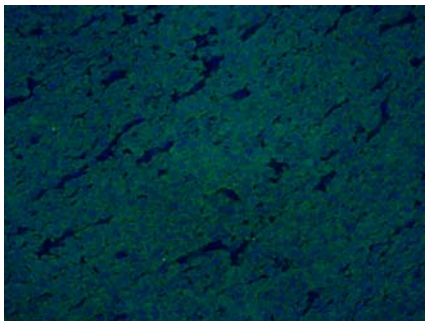


Figure 7. LNCaP + UGSM2-Gli2 tumors were immunohistochemically stained for GFP (green) and counterstained with DAPI (blue) to mark nuclei. UGSM2-Gli2 cells express GFP in addition to Gli2. Tumors that formed are composed of UGSM2-Gli2 stromal cells, and no LNCaP cells (no GFP). Therefore these tumors are sarcomas and not typical LNCaP carcinomas.

Since Gli2 induces UGSM2 cells to become tumorigenic, we cannot determine if UGSM2-Gli2 cells induce LNCaP carcinoma growth. LNCaP tumors take 3-4 weeks to begin forming and UGSM2-Gli2 sarcomas grew to a large size so that all of these mice had to be sacrificed within 2 weeks. Since these methods did not work, we used another methods of increasing Shh signaling in UGSM cells to determine if growth acceleration can be achieved by stromal Shh signaling.

Gli3-/-: Gli3 is a negative regulator of Shh signaling and loss of Gli3 in stromal cells increases Shh signaling independent of Shh ligand. We isolated prostate stromal cells from mice with a genetic defect that produces a null Gli3 allele (Gli3xt mice). These cells were isolated in the same fashion as UGSM-2 cells (see Shaw 2006). UGSM-Gli3-/- cells have an increase in the Shh transcriptional product Gli1 and a further increase if treated with Shh ligand.

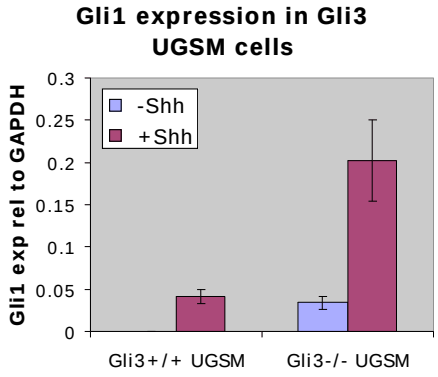


Figure 8. UGSM-Gli3+/+ or UGSM-Gli3-/- cells were treated with or without Shh ligand for 48 hrs. We analyzed Gli1 expression by real-time RT-PCR. Gli1 expression and Shh signaling is similar in Gli3-/- cells as to Gli3+/+ cells treated with Shh, revealing that Shh signaling in Gli3-/- cells is activated independent of Shh ligand.

We co-injected LNCaP + UGSM-Gli3+/+ (WT tumors) or LNCaP + UGSM-Gli3-/- and examined growth rates of tumors. LNCaP + UGSM-Gli3-/- tumors grow at a faster rate than LNCaP + UGSM-Gli3+/+ tumors. This indicates that stromal Shh signaling can induce tumor growth rate in the absence of Shh ligand. This supports that Shh tumor growth is due to a paracrine response from tumor stromal cells.

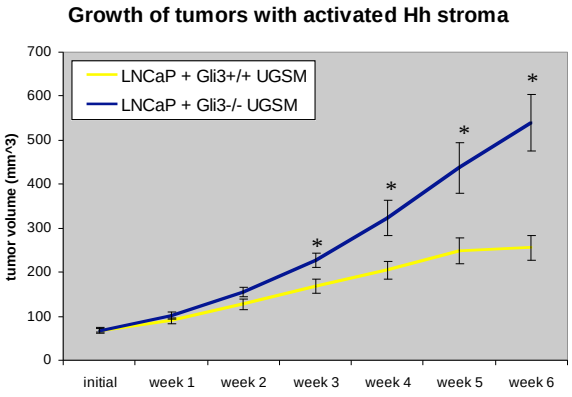


Figure 9. LNCaP were co-injected with UGSM-Gli3+/+ or UGSM-Gli3-/- cells and tumors were measured. Average tumor sizes per week after tumors were noted are shown. Student's t-test revealed significant differences at weeks 3-6. Analysis of slopes of individual tumors also showed a difference in the growth rate of tumors between the two groups.

c. Determine if stromal Gli1 or Gli2 is necessary for Shh-accelerated tumor growth. (Months 1-12)

In vitro: will not be done (see 1a, in vitro)

In vivo: We have included UGSM-2 cells in LNCaP xenograft tumors. Analysis of bi-clonal xenografts made with UGSM-2 cells stably expressing GFP shows that UGSM2-GFP cells comprise about 10% of the total stromal cells (UGSM-2 cells and host mouse stromal cells) within a xenograft tumor. We have concluded that loss of Gli1 or Gli2 in 10% of stromal cells is unlikely to exhibit a measurable effect on tumor growth.

Gli1-/- nude mice: We had suspected this may occur and we suggested that we could analyze tumor growth in Gli1-/- nude mice as an alternative to examine this aim. We have decided against taking this approach. Loss of Gli1 function is unlikely to alter tumor growth since recent studies have shown that Gli2 is the primary mediator of Hedgehog signaling in cells and Gli1 functions to amplify Gli2 activation (Lauth 2007). Since loss of Gli2 is embryonic lethal, this experiment would require an inducible Gli2 knockout mouse that has not been generated. This aim will cannot be completed with currently available mouse models.

Task 2. Identify Shh stromal target genes in prostate cancer. (Months 8-24)

a. Validate stromal target genes by cell-based assay. (Months 8-12)
We have previously completed a microarray study to identify Shh target genes in UGSM-2 cells. This task will validate the identified targets by RT-PCR analysis.

	UGSM-2 Cells		
Gene	Array w/ Shh	Validation w/ Shh	w/ Shh& Cyclopamine
Gli1	17.1	621	2.31
Ptc1	8.6	34	0.74
BRAK	27.6	18.4	0.48
Dner	24.9	15.87	0.26
Fgf5	9.1	22.56	1.56
Map3k12	3.97	0.96	1.06
Timp3	3.81	3.26	0.45
Angpt4	3.51	9.44	0.91
Hsd11b1	3.48	12.58	0.48
Tnmd	3.34	2.58	0.62
Artn	2.67	1.45	0.59
Fbn2	2.55	3.08	0.63
Igfbp-3	2.51	3.02	0.24
Ntrk3	2.4	0.9	0.95
Tiam1	2.3	3.16	0.18
Igfbp-6	2.28	2.83	1.31
Sod3	2.26	3.5	0.46
Plxna2	2.2	2.69	0.86
Sos1	2.04	0.9	0.9
Inhbb	1.03	2.08	0.84
Spdy	0.5	1.01	0.39
Rgs4	0.44	0.49	0.6
Fkbp1a	0.41	1.14	0.97
Sufu	0.37	0.97	0.64
Mmp13	0.21	0.28	0.23
Dmp1	0.16	0.4	2.42

Table 1. Shh target genes were analyzed by microarray analysis, RT-PCR validation and inhibited with cyclopamine. We identified 24 targets by array, validated 17 by RT-PCR and 16 of these are inhibited by cyclopamine.

Microarray analysis identified 24 target genes. 17 of these targets were validated using RT-PCR. We furthered this analysis by determining which of these genes is altered when cells are treated with the Shh specific antagonist cyclopamine. 16 of the 17 genes is reversed by cyclopamine treatment, indicating that these targets are specific to Shh.

b. Identify stromal target genes up-regulated by Shh signaling in LNCaP xenograft tumors. (Months 8-16)
The 16 validated target genes from 2a were analyzed in LNShh tumor stroma by species-specific RT-PCR. We also analyzed Hes1, a Shh target gene we have recently identified in prostate stroma.

Gene	LNShh tumors	Gli3-/- cells
Gli1	increase	increase
Ptc1	increase	increase
Angpt4	increase	decrease
Artn	no change	ND
BRAK	increase	no change
Dmp1	no change	ND
Dner	no change	increase
Fbn2	increase	ND
Fgf5	increase	no change
Hes1	increase	increase
Hsd11b1	increase	decrease
Igfbp-3	no change	ND
Igfbp-6	increase	increase
Ntrk3	no change	ND
Plxna2	no change	ND
Sod3	no change	ND
Tiam1	no change	ND
Timp3	increase	increase
Tnmd	no change	ND

Table 2. 17 Shh target genes and 2 canonical genes were analyzed in LNShh tumor stroma and in UGSM-Gli3 cells. Data illustrated shows if genes are changed in LNShh tumors relative to LNCaP tumors (middle column) or are changed in UGSM-Gli3-/- cells relative to UGSM-Gli3+/+ cells in culture (last column).

8 of the 17 target genes are modified in LNShh tumor stroma. Of these genes, 3 are also changed in Gli3-/- cells that accelerate tumor growth. Some of these genes may be identified as paracrine targets that increase tumor growth.

c. Determine whether identified Shh stromal target genes are expressed in human prostate cancers and determine whether expression correlates with level of Shh signaling. (Months 12-24)

Target genes in human tumors: We have begun to analyze expression of the target genes from 2b in human prostate cancer and histologically benign tissue from the same patient by RT-PCR. We have analyzed expression in 23 patients. First, we compared expression in tumor tissues versus benign tissue. This showed that neither Shh nor Gli1 are significantly increased in cancer relative to benign tissue. This result is consistent with our previous observation (Fan 2004). Of the target genes decribed in 2b above, BRAK is the only gene that is significantly increased in cancer relative to benign prostate. This has been previously noted (Schwartze 2005) and he noted that there is significant BRAK expression in prostate stromal cells. BRAK over-expression in prostate cancer cells slowed growth of tumors. Since Shh and Gli1 are not significantly altered in prostate cancer, we were not expecting to see significant differences in the stromal gene signature noted in 2b. We hypothesized that differences in the stromal phenotype of prostate tissue will change the Shh stimulated gene expression response of prostate stroma. One of the known changes in prostate stroma is the reactive stroma phenotype that has been shown to predict prostate cancer progression. Prostate with higher volumes of reactive stroma are more likely to recur after radical prostatectomy and

decrease survival (Ayala 2003). A recent study showed that reactive stroma predicts prostate cancer recurrence independent of Gleason grade and PSA (Yanagisawa 2007). The samples we analyzed were unique in that we had frozen tissue for RNA analysis as well as formalin-fixed paraffin embedded (FFPE) tissue. We stained the FFPE tissue by immunohistochemistry for 2 markers of reactive stroma: smooth muscle actin and vimentin. These markers show the location of myofibroblasts, cells that stain with both markers, that are typical exhibits of reactive stroma (Tuxhorn 2002). The amount of myofibroblasts was counted relative to total stroma and related to gene expression in individual samples. Again, we found that BRAK is over-expressed in reactive tumors relative to non-reactive tumors, reactive benign and non-reactive benign tissue.

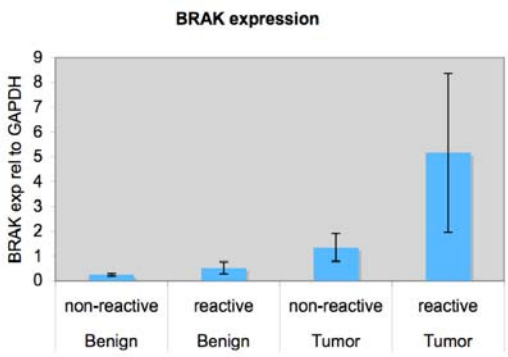


Figure 10. Expression of BRAK as human prostate is classified as tumor/benign and reactive/non-reactive. There is significantly more BRAK expression in tumors and reactive stroma further increases BRAK.

Correlation: To determine if expression of Shh/Gli1 relates to expression of the target genes, we performed Pearson's product-moment correlation analysis. Analysis of all samples together shows that Shh correlates with Gli1, Gli2 and Igfbp6. This shows that Shh expression per tissue sample correlates with Gli1, Gli2 and Igfbp6 expression and suggests that Shh controls transcription of these genes. Interestingly, Shh signaling is not unique to prostate cancer and is present in benign prostate tissue as well. However, this could also exhibit Shh signaling in prostates with cancer since the benign samples were also taken from a prostate with cancer, but do not exhibit histological signs of cancer. As the samples are grouped according to benign or tumor status, we found that Shh, Gli1, Gli2, ADAM12, Agpt4, HSD11b1, Igfbp6 and Timp3 correlate in tumor tissue. In benign tissue, Shh, Gli1, Gli2 and Igfbp6 are correlated. This suggests that Shh regulates expression of the unique genes ADAM12, Agpt4, HSD11b1, and Timp3 in cancer tissue, but not in benign tissue. This may relate to the increased growth of tumors since these 4 genes were originally identified in the LNShh xenografts wherein Shh drives tumor growth. It was curious that other target genes from the LNShh tumor stroma were not correlated with Shh or Gli1 in human tumors. To analyze

the effect of reactive stroma on correlation of target genes, we further classified the tissue samples according to reactive or non-reactive status. This showed an astounding correlation of all (Shh, Gli1, Gli2, Ptc1, Hip, ADAM12, Agpt4, BRAK, Fbn2, Fgf5, Hes1, HSD11b1, Igfbp6, Timp3) of the target genes in tumor reactive samples, that is not seen in the tumor non-reactive, benign non-reactive or benign reactive sample groups. This is the first analysis showing that stromal phenotype can regulate transcriptional pathways in prostate cancer. This is a unique result that shows that gene expression may not be altered in reactive tumors, but the pathway regulating gene expression of these tumors is indeed altered.
Proliferation: Since reactive status alters expression of the target genes we identified in LNShh xenografts, we examined proliferation in the same samples we analyzed gene expression and reactive stroma in by Ki67 immunohistochemistry. We found that proliferation is increased in reactive tumors, but these tumors have extremely variable proliferation and the difference is nearly significantly different ($p=0.0502$). It is extremely rare to find these type of human prostate samples: matched benign and tumor from the same patient, frozen tissue for RNA analysis and matching FFPE tissue blocks. Collection of additional samples is warranted. These data shows that human prostate cancer with a reactive stroma is likely to have an increased proliferation rate and this may in part explain the ability of these tumors to recur after radical prostatectomy that Rowley and Ayala have noted (Tuxhorn 2002, Ayala 2003).

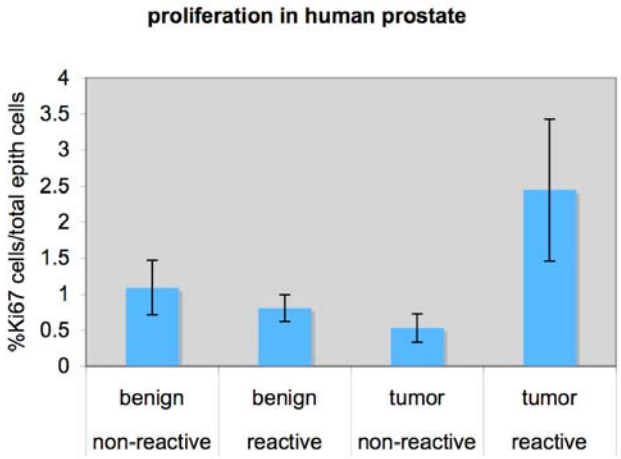
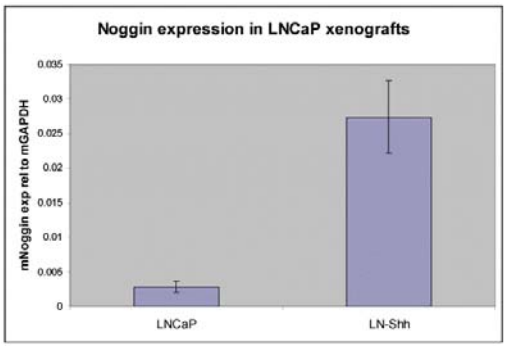


Figure 11: Analysis of proliferation in human prostate tumors by Ki67 immunohistochemistry. The results show Ki67 staining relative to total cells. Samples are separated according to their tumor/benign and reactive/non-reactive status.

Noggin: Before the microarray analysis was completed, I analyzed expression of Noggin, a gene whose expression correlates with Gli1 in embryonic prostate stroma. Noggin expression is increased in LNShh tumor stroma compared to LNCaP tumor stroma, and Noggin expression correlates with Gli1 in LNShh tumor stroma.



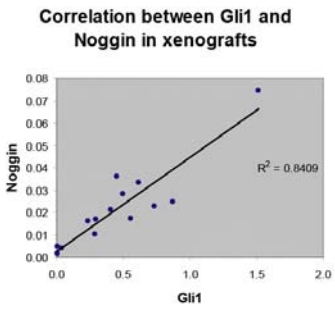
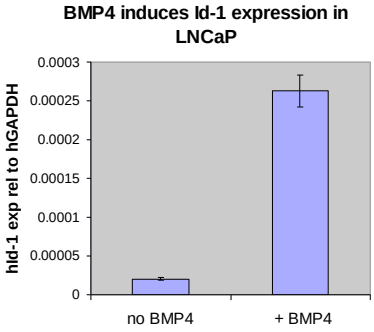


Figure 12. (left) Expression of Noggin mRNA was quantitated by real-time RT-PCR in LNCaP and LNShh tumors. (right) Expression of Noggin correlates with Gli1.

The function of Noggin is to antagonize BMP ligand effects. I examined BMP signaling by measuring the BMP transcriptional target Id-1 in LNCaP and LNShh tumors and found that BMP signaling is reduced in LNShh tumors.



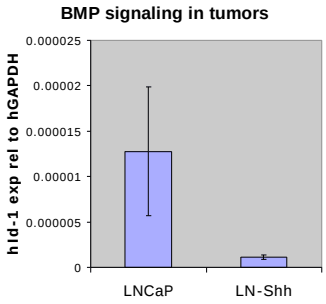


Figure 13. Id-1, a transcriptional product of BMP signaling was analyzed in LNCaP in culture and in tumors. (left) LNCaP were treated with BMP-4 for 48 hours and human Id-1 was measured by RT-PCR. (right) LNCaP and LNShh tumors were analyzed for Id-1 mRNA.

BMP is cited to inhibit proliferation of prostate cell lines and BMP inhibits LNCaP. Noggin addition reverses the inhibitory activity of BMP.

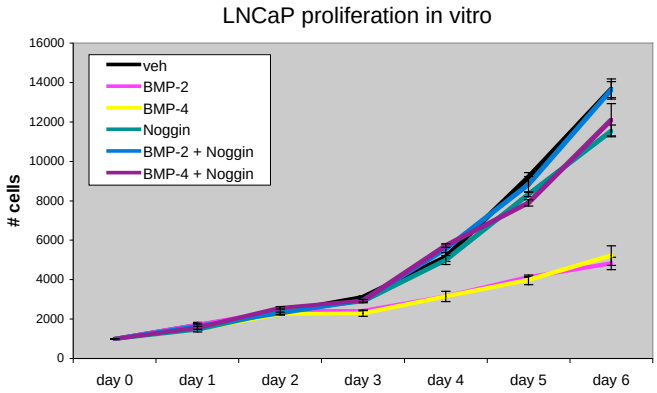


Figure 14. LNCaP in culture were treated with BMP-2, BMP-4, Noggin or combinations of BMP + Noggin. Cell growth was measured using a Coulter counter.

Since Noggin inhibits BMP signaling and BMP signaling inhibits LNCaP proliferation, Shh-induced Noggin may increase LNShh tumor growth. Noggin is a likely Shh-induced target gene in tumor stroma that may regulate prostate tumor growth.

Task 3. **Test the effect of individual stromal target genes on tumor growth. (Months 18-36)**

a. Achieve OE of selected stromal target genes in UGSM-2 cells. (Months 18-26)

Noggin cDNA was obtained and cloned into a retroviral vector. Noggin was stably overexpressed in UGSM-2 cells. Flow cytometry was used to isolate cells expressing the Noggin-IRES-GFP genes.

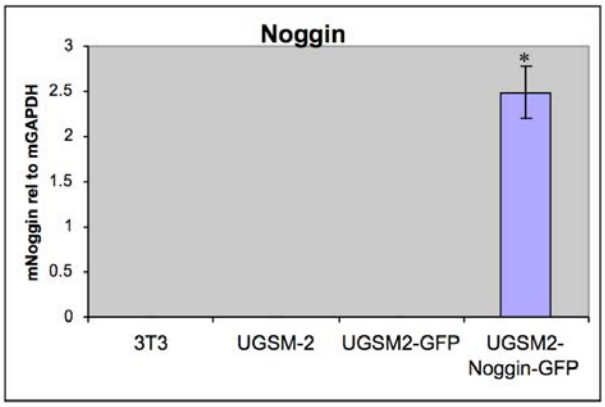


Figure 15. Noggin expression in different cell types by RT-PCR. Noggin expression is increased >1000-fold in UGSM2-Noggin cells.

Conditioned media from UGSM2-Noggin cell line inhibits BMP inhibition of LNCaP growth. This indicates that Noggin is secreted by UGSM2-Noggin cells and is functional in blocking BMP activity.

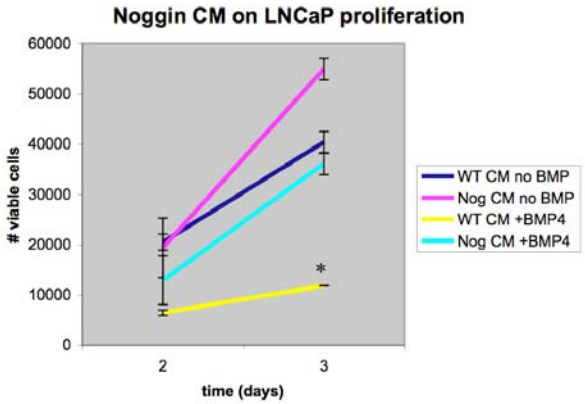


Figure 16. Conditioned media was prepared from UGSM2-Noggin or UGSM2-GFP cells by incubation with medium containing 1% FCS for 48 hours. CM and/or BMP-4 was added to LNCaP cells, and cell growth was monitored daily using a ViCell counter that counts viable cells.

b. Assay effect on tumor cell proliferation. (Months 26-36)

In vitro: will not be done (see 1a, in vitro)

In vivo: LNCaP/LNShh cells were co-injected with UGSM2-Noggin or UGSM2-WT cells. Tumor growth rates were monitored.

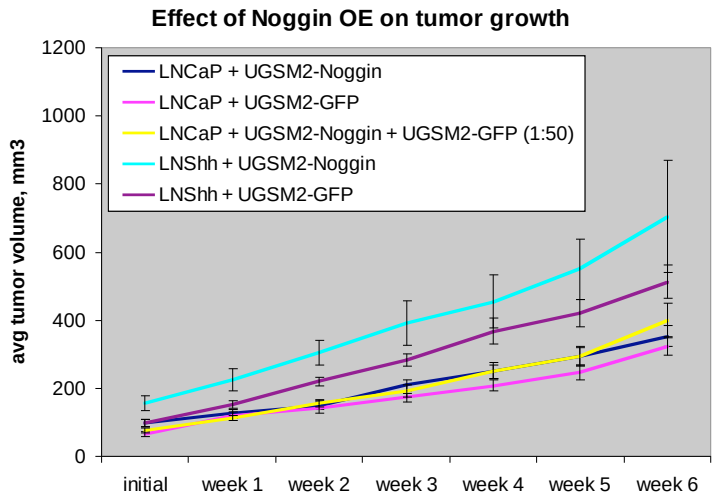


Figure 17. Tumor growth effects of stromal Noggin. LNShh tumors grow faster than LNCaP tumors. Noggin expression does not effect growth rates of tumors, regardless of Shh being expressed.

Noggin overexpression did not alter tumor growth rates in the presence or absence of Shh. UGSM2 cells form a minority of the total stroma in tumors, so we examined Noggin expression in tumors. The level of Noggin expressed here is 20-fold more than the level seen in LNShh tumor stroma without UGSM2-Noggin cells. Therefore Noggin is upregulated above the level seen in LNShh tumor stroma when UGSM2 stroma is present.

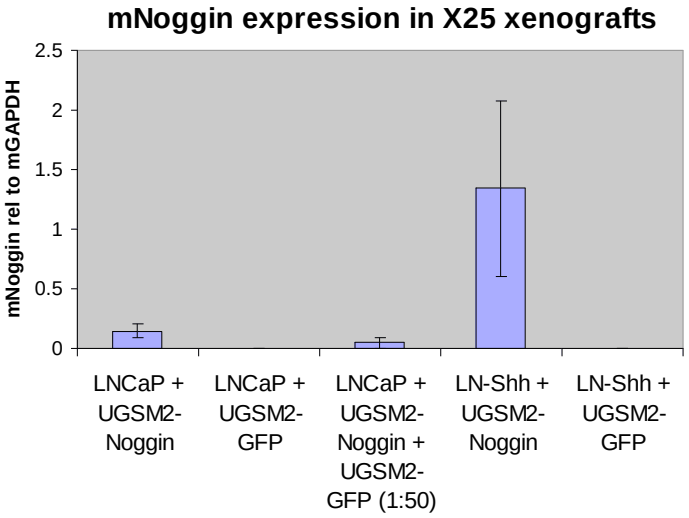


Figure 18. Expression of mouse Noggin in tumors by RT-PCR.

BMP signaling was reduced by Noggin OE in tumors, verifying that Noggin was functional. Despite the loss of BMP signaling in Noggin OE tumors, this did not modify tumor growth rate.

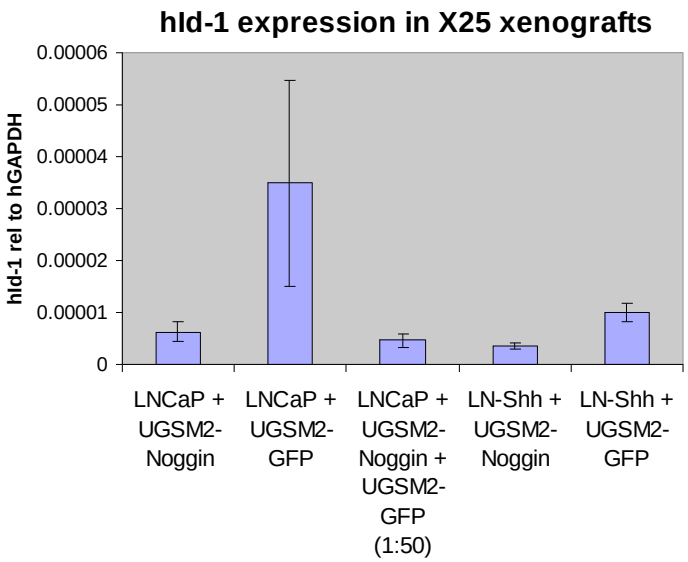


Figure 19. Id-1 expression (BMP signaling) in tumors by RT-PCR.

c. Examine effect of loss-of-function. (Months 18-36)

In vitro: will not be done (see 1a, in vitro)

In vivo: This experiment will not be completed. Stable expression of RNAi construct for long enough times to examine effects months later in xenografts is not successful and in vivo toxicity is common (Snove 2006). Also, co-injection of UGSM2 with LNCaP results in a minority of UGSM2 cells amongst stromal cells in the tumor. This is a large complication for loss-of-function (LOF) studies since wild-type stromal cells will compensate the LOF UGSM2 cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Shh growth effect is mediated by paracrine signals
- Cannot demonstrate if Shh growth effect is mediated by either soluble or insoluble secreted factors from tumor stromal cells (co-cultures)
- Blockade of autocrine Hedgehog signaling in LNShh cells, with dominant negative Gli2, does not block Shh-induced tumor growth
- Gli1 or Gli2 overexpression in tumor stroma accelerates does not alter tumor growth in the absence of Shh
- Gli3 loss of expression in tumor stroma accelerates tumor growth in the absence of Shh. Stromal Shh signaling increases tumor growth.
- Noggin is upregulated in stroma of LN-Shh tumors
- Noggin induces growth of LNCaP by blocking the inhibitory actions of BMP2/4
- Recently completed microarray to identify genes whose expression is altered by Shh treatment of UGSM-2 cells.
- Validated 16 genes that are inhibited by cyclopamine
- 10 target genes are modified in LNShh tumor stroma
- There is no increase in Shh, Gli1 expression in human prostate tumors relative to benign tissue taken from the same prostate
- Of the target genes, BRAK is the only target gene that shows increased expression in tumors compared to benign prostate
- Shh signaling is present in prostate tissue, regardless of benign or tumor state
- Shh signaling elicits expression of ADAM12, Agpt4, HSD11b1 and Timp3 in tumors, but not in benign tissue
- Human prostate tumors with a high amount of reactive stroma have correlated expression of Shh and all of the target genes identified in mouse xenografts
- Human prostate tumors with a high amount of reactive stroma have an

increased proliferation rate

- Noggin overexpression in tumor stroma cannot accelerate tumor growth in the absence of Shh

- Noggin overexpression in tumor stroma does not increase tumor growth in addition to Shh

- Noggin overexpression reduces BMP signaling in LNCaP, but does not modify tumor growth rate

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Manuscripts

Cook C, Vezina CM, Hicks SM, Shaw A, Yu M, Peterson RE, Bushman W (2007) Noggin is Required for Normal Lobe Patterning and Ductal Budding in the Mouse Prostate. Dev Biol, 312(1): 217-230.

Bruxvoort KJ, Charbonneau HM, Giambernardi TA, Goolsby JC, Qian C-N, Zylstra CR, Robinson DR, Roy-Burman P, Shaw AK, Buckner-Berghuis BD, Sigler RE, Resau JH, Sullivan R, Bushman W, Williams BO (2007) Inactivation of Apc in the Mouse Prostate Causes Prostate Carcinoma. Cancer Res, 67(6): 2490-2496.

Zhang J, Lipinski R, Shaw A, Gipp J, Bushman W (2007) Lack of Demonstrable Autocrine Hedgehog Signaling in Human Prostate Cancer Cell Lines. J Urol, 177: 1179-1185.

Shaw A, Bushman W (2006) Isolation and Characterization of an Immortalized Mouse Urogenital Sinus Mesenchyme Cell Line. The Prostate, 66(13): 1347-1358.

Invited Review

Shaw A, Attia S, Bushman W. (2008) Prostate stromal and urogenital sinus mesenchymal cell lines for investigations of stromal-epithelial interactions. Differentiation (in press)

Shaw A, Bushman W (2006) Hedgehog Signaling in the Prostate. J Urol, 177: 832-838.

Abstracts

Shaw A, Gipp J, Bushman W (2007) Hedgehog Signaling Regulates Prostate Tumor Growth by a Paracrine Mechanism. Poster at The 4th International Conference on Tumor Microenvironment, Florence, Italy

Shaw A, Bushman W (2006) Effect of Hedgehog Signaling on Tumor Growth is Influenced by Stromal Composition. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Gipp J, Bushman W (2006) Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Fan L, Wellner M, Gipp J, Bushman W (2006) Hedgehog Signaling and Androgen Independent Prostate Tumor Growth. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Gipp J, Bushman W (2006) Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth. American Association for Cancer Research Annual Meeting, Washington, DC

Shaw A, Fan L, Wellner M, Gipp J, Bushman W (2006) Hedgehog Signaling and Androgen Independent Prostate Tumor Growth. American Association for Cancer Research Annual Meeting, Washington, DC

Oral Presentations

Karolinska Institutet, March 12, 2007, Stromal-Epithelial Hedgehog Signaling in Prostate Cancer, Novum, Huddinge, Sweden

Vanderbilt-Ingram Cancer Center, February 16, 2007, Stromal-Epithelial Hedgehog Signaling in Prostate Cancer, Nashville, TN

Tufts University, February 9, 2007, Stromal-Epithelial Hedgehog Signaling in Prostate Cancer, Boston, MA

University of California-San Francisco, January 18, 2007, Stromal-Epithelial Hedgehog Signaling in Prostate Cancer, San Francisco, CA

American Association for Cancer Research Annual Meeting, April 1-5, 2006 "Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth" Washington, DC

American Association for Cancer Research Annual Meeting, April 1-5, 2006 "Hedgehog Signaling and Androgen Independent Prostate Tumor Growth" Washington, DC

UW Comprehensive Cancer Center Prostate Research Group, February 3, 2006 "Hedgehog and Bone Morphogenetic Protein Signaling in Prostate Cancer" Madison, WI

Licenses

We have been issued licenses for UGSM cell lines through Wisconsin Alumni Research Foundation

Development of cell lines

UGSM-2 cell line and derivatives of the cell lines: UGSM2-SmoM2, UGSM2-Gli1, UGSM2-Gli2, UGSM2-Noggin

UGSM-Gli3-/- and UGSM-Gli3+/+ cell lines

Animal Models

I have developed the bi-clonal xenograft model by co-injection of LNCaP cells with UGSM cells. Both components of the bi-clonal model can be genetically modified to analyze tumor-stromal interactions.

CONCLUSION:

This research is the first molecular analysis of a biphasic stromal-epithelial route driving prostate tumor growth. We have established that Sonic hedgehog drives tumor growth by a paracrine mechanism in xenograft tumors. We have isolated several stromal paracrine products that may be involved in inducing tumors to grow. We have shown that the stromal phenotype in addition to the presence of carcinoma epithelial cells dictates the transcriptional program to Shh. Our analysis of the correlation of gene expression with reactive stroma diagrams a novel technique for examining gene expression in tumors by showing that tumors with correlated Shh gene expression have a reactive stroma and grow rapidly. Since reactive prostate tumors have a higher rate of recurrence after prostatectomy, the correlation of Shh signaling and the set of target genes that were identified

in rapidly growing xenograft tumors may allow us to predict the insidious prostate tumors that are likely to recur from the tumors that are cured after surgery. The gene program identified here may provide appropriate therapeutic targets.

Future studies should examine the effect of diminishing Shh signaling in tumors. New inhibitors such as the small molecules GANT61 and GANT58 inhibit Gli1/2 function and should be examined using in vivo mouse xenografts. Another important aspect of this research is to examine prostate cancer progression or recurrence with respect to Shh signaling. The model we used, subcutaneous LNCaP xenografts, is not a prostate cancer cell line that progresses. These grafts never metastasize or invade. Our research has shown that Shh signaling may predict cancers that progress. Future studies should examine Shh signaling in a prostate cancer model that can progress, such as LNCaP orthotopically implanted that can metastasize to the lymph node, lung and bone. I suspect that the increased proliferation of LNCaP cells when Shh is present allows the cells to develop metastatic tumors. Another interesting topic is to examine the link between reactive stroma and Shh signaling. Shh is a developmental pathway and my studies have shown that myofibroblasts are present in the developing prostate during early ductal morphogenesis. This suggests that Shh and reactive stroma/myofibroblasts are used during times of enhanced epithelial growth and differentiation. Altered homeostasis occurs during development and during carcinogenesis and this links development to cancer biology. Further understanding of how Shh signaling is linked to myofibroblasts during prostate development and likely to lead to a better understanding of how Shh-myofibroblasts are involved in prostate cancer.

I have completed by PhD and have begun a postdoctoral position at Vanderbilt University in the laboratory of Harold L. Moses. I defended my thesis in October 2007 and graduated in December 2007. In my new lab I will be able to continue studying the role of stromal cells in prostate and breast cancer progression. I am currently writing 2 papers in description of the results of these studies.

APPENDIX:

Shaw A, Attia S, Bushman W. (2008) Prostate stromal and urogenital sinus mesenchymal cell lines for investigations of stromal-epithelial interactions. Differentiation (in press)

Differentiation

Prostate stromal and urogenital sinus mesenchymal cell lines for investigations of stromal-epithelial interactions

Journal:	*Differentiation*
Manuscript ID:	DIFF-2007-0214.R2
Manuscript Type:	Invited Review
Date Submitted by the Author:	17-Feb-2008
Complete List of Authors:	Shaw, Aubie; University of Wisconsin-Madison, Surgery Attia, Steven; University of Wisconsin-Madison, Medicine Bushman, Wade; University of Wisconsin-Madison, Surgery
Keywords:	mesenchyme, cell line, prostate, UGSM-2

TITLE

Prostate stromal and urogenital sinus mesenchymal cell lines for investigations of stromal-epithelial interactions

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ABSTRACT

Bidirectional signaling between the urogenital sinus epithelium and mesenchyme is an essential element of prostate development that regulates ductal morphogenesis, growth and differentiation. Comparable interactions between the epithelium and stroma in the adult prostate appear to regulate normal growth homeostasis. Alterations in the stromal-epithelial dialogue that recapitulate features of the mesenchymal-epithelial interactions of development may play a critical role in the development of benign prostatic hyperplasia and in the progression of prostate cancer. For this reason, the mesenchymal-epithelial interactions of development are of considerable interest. In this review, we provide an overview of the mesenchymal contribution to rodent prostate development with an emphasis on the stage just prior to ductal budding (embryonic day 16; E16) and describe the isolation, characterization and utility of a newly established E16 urogenital sinus mesenchymal cell line.

KEY WORDS

UGSM-2; cell line; prostate; mesenchyme

Viewed through a dissecting microscope, as when performing tissue separation for the purposes of a recombination experiment (Staack et al., 2003), the mesenchyme of the developing rodent prostate is all the "stuff" that isn't epithelium (Figure 1). What is this stuff? Where did it come from? And, what does it do?

At embryonic day sixteen (E16), just prior to the onset of ductal budding, the fetal rodent prostate consists of a multilayered epithelial tube cloaked by an arrangement of mesenchymal cells. Urogenital epithelium (UGE) is derived from the embryonic endoderm that is separated from the hindgut when the cloaca is divided by the urogenital septum (Staack et al., 2003). Urogenital mesenchyme is derived from splanchnic mesoderm that is thought to originate from cells migrating through the primitive streak. Histologic examination of the E16 urogenital sinus (UGS) reveals a lumen surrounded by a multilayered epithelium in turn surrounded by a collar of mesenchymal cells. Cells immediately adjacent to the epithelial layer are designated the periurethral mesenchyme and studies in the rat show that mesenchymal androgen receptor (AR) expression first appears in this layer (Hayward et al., 1996). Additional mesenchymal cells reinforce the periurethral layer and, together, comprise a substantial mesenchymal layer of varying thickness that runs the length of the prostatic urethra. A distinct mesenchymal condensation overlying the site of ventral prostate budding is termed the ventral mesenchymal pad (VMP) (Timms et al., 1995); similar, less prominent, condensations appear over the sites of dorsolateral prostate budding. Although the VMP is not sexually dimorphic (Thomson et al., 2002), these areas of condensation correspond to regions of mesenchyme involved in extensive branching morphogenesis during postnatal prostate development. Tissue recombination experiments indicate that there is regional heterogeneity of the fetal mesenchyme's ability to induce prostate development and confer ventral versus dorsolateral lobe identity in the rodent (Timms et al., 1995). The basis for this regional specification is as yet unknown, although it has been suggested that Hox genes may play a role (Podlasek et al., 1999). The anterior prostate, also termed the coagulating gland, is unique with respect to its embryonic contributions. The buds of the anterior prostate arise from the UGS, like the buds of the ventral and dorsolateral prostate, but postnatal branching morphogenesis of the anterior prostate occurs within the mesodermal sheath of the seminal vesicle (Podlasek et al., 1999).

The E16 mesenchyme contains myofibroblasts that coexpress vimentin and smooth muscle α-actin (SMA). Myofibroblasts are also found in the human prenatal prostate at weeks 23-25 (Bierhoff et al., 1997) and are thought to be precursors of the fibroblasts and smooth muscle cells that dominate the adult prostatic stroma. Detailed studies of stromal differentiation in the developing rat prostate showed that differentiation of mesenchymal cells to smooth muscle that surrounds the mature ducts occurs in the postnatal prostate in a proximal-distal fashion as ductal morphogenesis proceeds (Hayward et al., 1996). Myofibroblasts are present in the adult prostate at sites of benign prostatic hyperplasia (BPH) and in prostate carcinoma (Bierhoff et al., 1997; Ayala et al., 2003). Evaluation of the roles of these cells in the fetal prostate may explain any role they may have in adult prostate pathology.

Recent studies in a variety of organs and tissues have identified tissue-specific progenitor cells that exhibit enormous proliferative potential and the ability to differentiate into one or more terminally differentiated cell types. There are many questions regarding the origin of the adult prostate stroma since this aspect of prostate stromal biology has not been well studied. Is there a stromal stem/progenitor cell present in the adult prostate? If a stromal stem cell exists, is it separate from the epithelial stem cell, or is there a single stem cell that generates both the stromal and epithelial

populations? Lin et al. cultured stromal cells derived from patients with BPH. These cells express several markers of mesenchymal stem cells (MSC) and can differentiate into smooth muscle, adipocyte and osteogenic cell lineages (Lin et al., 2007). This suggests that at least part of the adult prostate stroma arises from MSC. It is important to identify the progenitor cell(s) for the adult stroma and determine the possible contribution of bone marrow cells to the adult stroma. Neoplasia or BPH may stimulate proliferation or migration of stromal progenitors or MSC. We are not aware of any published studies that have looked for the presence of stem or primitive progenitor cells in the UGS mesenchyme and/or examined their role in postnatal stromal proliferation and smooth muscle differentiation. The fetal mesenchyme may also contain the precursors of vascular and lymphatic components of the adult prostate; however, the ontogeny of vascular development in the prostate has not been studied and their precursors have not been clearly identified. Indeed, intensive efforts to characterize ductal budding and epithelial differentiation during prostate development stand in striking contrast to the relative lack of understanding of mesenchymal proliferation and differentiation during generation of the adult prostatic stroma. Tissue recombination of human prostate epithelial cells with rat UGM showed extensive smooth muscle sheet differentiation, which is characteristic of the human, but not the rodent, prostate. This reveals that epithelial cells control differentiation of prostate mesenchyme in a species-specific manner (Hayward et al., 1998). Paracrine interactions of epithelium and mesenchyme are clearly an area in need of further study.

Mesenchymal-/stromal- epithelial interactions are a pivotal aspect of prostate development, differentiation, normal growth regulation and neoplasia. The mesenchyme is the site of expression of many critical regulators of ductal budding and development including transforming growth factor-β, fibroblast growth factor (FGF-10, FGF-7), bone morphogenic protein (BMP-4, BMP-7), Noggin, secreted frizzled related protein 1 and several Hox genes (Tomlinson et al., 2004; Grishina et al., 2005; Marker et al., 2003; Joesting et al., 2005; Cook et al., 2007). Urogenital sinus mesenchyme (UGM) expresses androgen receptors (Thompson et al., 1986) and participates in the metabolism of steroid hormones (Neubauer et al., 1985). The stroma contains aromatase (Ellem et al., 2004) and 5-α-reductase (Cowan et al., 1977), enzymes important for converting testosterone to estradiol or dihydrotestosterone, respectively. Estrogen and androgen are important for prostate growth, development, carcinogenesis and hyperplasia and our understanding of steroid effects in homeostasis may explain aberrant roles in the adult prostate (McPherson et al., 2007; Prins et al., 2007; Cunha et al., 2004). UGM is the target of regulation by signaling molecules secreted by the epithelium such as Sonic Hedgehog (Shh), Indian Hedgehog and insulin-like growth factor-1. Several investigators have performed transcriptional profiling of rat or mouse prostate mesenchyme (Vanpoucke et al., 2007; Zhang et al., 2006). These studies identified new pathways controlling prostate development, but further understanding of the biology requires the use of mesenchymal cell lines. Such cell lines allow deletion or activation of specific signaling pathways in vitro and in vivo to study the biological effect of these perturbations. An ideal mesenchymal or stromal cell population would have the following properties: immortal, genetically stable, responds to known biochemical mediators, gene expression that is similar to freshly isolated tissue, allows in vivo and in vitro modeling and can be genetically modified.

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Several immortalized prostate stromal cell lines exist: NbF-1, RSPC-2T, PS-1, WPMY-1, PS30, S2.13, and PM151T (Table 1). These cell lines have been evaluated with respect to androgen signaling and smooth muscle differentiation because these properties are important for prostate carcinoma studies and for description of the stromal

cell type, respectively. Human prostate stromal cells were immortalized using large T antigen, HPV16 E6/E7 or telomerase catalytic subunit. Large T antigen and HPV16 E6/E7 use the p53 or pRb pathway to prevent senescence and can contribute to future genetic anomalies in the cell line because of reduced gene surveillance. This is an important factor to consider when selecting an immortalized cell line. NbF-1 cells were derived from the ventral prostate of four adult rats. Dihydrotestosterone, but not testosterone, stimulates DNA synthesis in these cells (Chang and Chung, 1989). RSPC-2T cells were derived from 10-day-old rats and were immortalized with v-myc. These cells express SMA, but not AR (Hoeben et al., 1995). PS-1 cells are derived from the adult rat ventral prostate. PS-1 cells are smooth muscle cells that express desmin and SMA. PS-1 cells express AR and androgen enhances cellular proliferation (Gerdes et al., 1996). WPMY-1 cells were acquired from a 54-year old Caucasian male during cystoprostatectomy who had no evidence of neoplasia and immortalized with large T-antigen. WPMY-1 cells express SMA, vimentin and fibronectin, but not desmin and is therefore characterized as a myofibroblast cell line. WPMY-1 cells express AR and androgen increases proliferation (Webber et al., 1999). PS30 cells were derived by HPV16 E6/E7 immortalization of primary human prostate cells obtained from normal tissue at radical prostatectomy for localized prostate cancer. PS30 cells are myofibroblasts that express SMA, vimentin, but not desmin or heavy chain myosin. PS30 cells express AR, but androgen does not alter cellular proliferation (Price et al., 2000). S2.13 cells are clonal and derived from tissues from men aged 51-78 years via transurethral resection for BPH and immortalized with a temperature sensitive SV40 large T-antigen. S2.13 cells are fibroblasts that express vimentin, but not SMA or AR (Daly-Burns et al., 2007). PM151T cells were derived from the prostatic normal transition zone of a 62-year old man during radical prostatectomy performed for localized prostate cancer. Primary stromal cells were immortalized by overexpression of human telomerase catalytic subunit. Smooth muscle PM151T cells express SMA, calponin, heavy chain myosin and AR (Kogan et al., 2006).

These cells, derived from both rodents and humans, provide important models for study of stromal-epithelial interactions in the adult prostate. However, these are not suitable for study of mesenchymal roles in prostate development since these cells have differentiated and cannot be used to trace mesenchymal differentiation. These cell lines have not been tested for prostate inductive capacity or the ability to integrate normally into the developing prostate. These studies require a UGM line that is genetically stable, can be genetically manipulated, can participate in prostate development in vivo, is responsive to androgen, and models mesenchymal differentiation and signaling activities related to paracrine epithelial signals.

In this regard, three immortalized urogenital sinus mesenchyme cell lines are available: U4F, rUGM and UGSM-2 (Table 2). U4F cells were derived from an E18 male rat embryo organ culture. Mesenchymal cells emanated from the UGS tissue onto the culture dish and immortalized spontaneously. U4F cells express AR, have vimentin intermediate filaments but do not express desmin (Rowley, 1992). Another cell line, rUGM, was also derived from an E18 male rat embryo. This cell line expresses AR and vimentin, but not desmin (Zhau et al., 1994). Neither the U4F nor the rUGM cell lines have been tested in the in vivo prostate recombination model. The UGM cell line, UGSM-2, also expresses vimentin and AR and does not express desmin. The UGSM-2 cell line has been further characterized in vivo.

The UGSM-2 cell line is a clonal cell line generated from an *INK4a* mouse E16 embryo, a transgenic knockout mouse that lacks the $p16^{INK4a}$ and $p19^{ARF}$ proteins that trigger senescence. UGSM-2 cells express AR, vimentin, and SMA but not smooth

muscle markers desmin or heavy chain myosin (Shaw et al., 2006). UGSM-2 cells are myofibroblasts in culture. Their lack of mature smooth muscle markers is consistent with their origin from the prenatal urogenital sinus mesenchyme, since few mesenchymal cells express mature smooth muscle markers at E16 (Hayward et al., 1996). Not only do UGSM-2 cells express AR, but androgen treatment of UGSM-2 cells cultured in vitro changes the morphology of the cells and stimulates proliferation (Shaw et al., unpublished). The cells are stably tetraploid, contact inhibited and non-tumorigenic in vivo. These cells participate in prostate development as shown by the ability of UGSM-2 cells grafted together with E16 intact UGS (i.e., both UGM and UGE) to proliferate and become part of the periductal stroma in the mature prostate grafts (Figure 2). The capacity of UGSM-2 cells to differentiate into bona fide smooth muscle cells in vivo has not been fully characterized. A distinguishing feature of UGM tissue is its inductive capacity. Tissue recombination studies have shown that isolated mouse E16 UGM can induce prostate morphogenesis in epithelia derived from the UGS or adult bladder (Cunha et al., 2004). To screen for inductive capacity in UGSM-2 cells, we grafted UGSM-2 cells with isolated E16 UGS epithelium (separated from UGM) under the renal capsule of an adult male nude mouse. These grafts did not exhibit prostate formation (Shaw et al., 2006). However, since UGSM-2 cells can integrate into the developing prostate, UGSM-2 cells are a useful UGM cell line for studying prostate developmental pathways.

We have used the UGSM-2 cell line to study Hedgehog (Hh) signaling. During prostate development, Hh peptide is secreted by the epithelium and acts upon the adjacent mesenchymal cells to activate the intracellular Hh signaling pathway. Our studies showed that the UGSM-2 cells are Hh responsive and that Shh peptide induces expression of the conserved Hh target genes Patched-1 and Gli1, replicating what is observed in the freshly isolated UGM (Shaw et al., 2006). Using UGSM-2 cells, we performed a microarray analysis to identify Hh target genes in the developing prostate (Yu et al., unpublished). The majority of putative target genes examined in detail exhibited Hh-regulated expression in the intact UGS and/or in primary UGS mesenchymal cells. We have also used UGSM-2 cells to characterize the role of Noggin in prostate development to examine the effect of BMP-4 on mesenchymal Noggin expression (Cook et al., 2007). These examples illustrate the broad potential of this cell line to identify potential targets of regulation by signaling pathways and examine the actions of signaling factors and transcriptional regulators important in prostate development. Further, the androgen responsiveness of these cells permits studies examining the influence of testosterone on these interactions.

UGSM-2 cells grow as monolayers in cell culture and can be co-cultured with epithelial cells. We discovered that co-culture of UGSM-2 cells with BPH-1 prostate epithelial cells in two-dimensional culture resulted in congregation of human BPH-1 cells in acinar-like structures surrounded by UGSM-2 stromal cells (Figure 3). A similar localization was seen when the cells were grafted together under the renal capsule (Figure 3). These studies show that UGSM-2 cells interact with human BPH-1 epithelial cells by grouping cell types to resemble cell arrangements in solid epithelial cords seen in early prostate development. This provides a useful system to study mesenchymal-epithelial signaling pathways in vitro and in vivo.

We have stably labeled UGSM-2 cells with monomeric red fluorescent protein to enable visualization of the cells in vitro (Figure 4) and in vivo. This facilitates monitoring of live cells in co-cultures and allows location of the cells in vivo. Co-culture of UGSM-2 cells with BPH-1 cells engineered to over-express Shh results in activation of the Hh pathway and expression of Gli1 in UGSM-2 cells. Grafting of UGSM-2 cells engineered

to over-express the BMP antagonist Noggin with LNCaP tumor cells results in inhibition of BMP signaling in the xenograft or in culture (Shaw et al., unpublished). In both instances, the use of species-specific RT-PCR primers permits the selective quantitation of gene expression changes in the human BPH-1 epithelial cells and mouse-derived stroma. This allows selective monitoring of stromal-epithelial signaling pathways in which Shh elicits Hh signaling in UGSM-2 cells, but not in BPH-1 cells (Shaw et al., 2006).

Since genetic manipulation of UGSM-2 cells may find use in a wide array of studies, we will point out several specific methodological considerations. As a consequence of being derived from the *INK4a* transgenic mutant created by insertion of a neomycin resistance gene, UGSM-2 cells are neomycin resistant. Plasmid vectors encoding hygromycin or zeocin resistance genes may be used for selection and maintenance of UGSM-2 cells in culture. However, UGSM-2 cells show a low efficiency of transfection using standard methods. We have found adenovirus vectors useful for transient expression studies and retroviral infection with vectors expressing green fluorescent protein selection to be most effective for generating stably transfected cells that can be sorted by fluorescence-activated cell sorting.

CONCLUSIONS

The UGSM-2 cell line possesses many features that make it useful to study the mesenchymal-epithelial interactions of prostate development: genetic stability, androgen responsiveness, and the ability to participate in prostate morphogenesis in vivo. Our studies of Hh signaling and Noggin regulation suggest that this cell line recapitulates the transcriptional and regulatory response of the UGS mesenchyme. However, no cell line can recapitulate in monolayer culture the microenvironment of mesenchymal cells in the fetal prostate and the regional variation in the identity, response and developmental fate of the UGS mesenchyme itself. For this reason, in vitro studies using these cell lines are best when complemented by in vivo studies to corroborate an in vitro observation and determine its biological significance.

ACKNOWLEGEMENTS

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Figure Legends

Figure 1. Isolation of UGSM-2 cells. The urogenital sinus of an E16 male INK4a-/- transgenic mouse embryo was subjected to collagenase digestion to separate epithelial (UGS-E) and mesenchymal (UGS-M) layers. UGS-M was placed into a culture dish and allowed to proliferate to confluence. Polyclonal UGSM cells were ring cloned to develop several stable clones, including the UGSM-2 cell line. Taken from Shaw A, Papadopoulos J, Johnson C, Bushman W. Isolation and characterization of an immortalized mouse urogenital sinus mesenchyme cell line. Prostate. 2006 Sep 15;66(13):1347-58.

Figure 2: UGSM-2 cell fate in development of the urogenital sinus (UGS). UGSM-2 cells labeled with BrdU were seeded with dissected UGS tissue and allowed to develop under the renal capsule of male nude mice. Immunohistochemistry shows UGSM-2 cells (red) in the periductal regions of mature prostate. Epithelial cells are marked with pan-cytokeratin (green). Taken from Shaw A, Papadopoulos J, Johnson C, Bushman W. Isolation and characterization of an immortalized mouse urogenital sinus mesenchyme cell line. Prostate. 2006 Sep 15;66(13):1347-58.

Figure 3: Interaction of UGSM-2 and human BPH-1 epithelial cells. (A) UGSM-2 cells and/or BPH-1 cells grown on rat tail collagen. Co-cultures show small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells (arrows). (B) UGSM-2 and/or BPH-1 cells renal capsule grafts after 4 weeks. Grafts of UGSM-2 cells alone yields only stromal tissue. BPH-1 cells grafted alone do not produce identifiable viable grafts. Co-grafting of UGSM-2 and BPH-1 cells results in small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells.

Figure 4: Labeling of cells enables tracking of cell proliferation, morphology and motility in co-cultures. UGSM-2 cells are stably labeled with monomeric red fluorescent protein and LNCaP cells are labeled with green fluorescent protein. This shows proliferation of each cell type over seven days in culture. Days 1, 3, 5 and 7 are shown. Taken from Shaw A, Papadopoulos J, Johnson C, Bushman W. Isolation and characterization of an immortalized mouse urogenital sinus mesenchyme cell line. Prostate. 2006 Sep 15;66(13):1347-58.

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Figure 1 is an eps file made using Adobe Illustrator CS2

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Table Legends

Table 1: Adult prostate stromal cell lines

Table 2: Urogenital sinus mesenchyme cell lines

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Table 1: Adult prostate stromal cell lines

Cell line	Phenotype	Species	How Immortalized	Markers	AR	Reference
RSPC-2T	smooth muscle/ myofibroblast	rat	v-myc	SMA	no	Hoeben 1995
PS-1	smooth muscle	rat	spontaneous	desmin, SMA	yes	Gerdes 1996
WPMY-1	myofibroblast	human	Tag	SMA, VIM, FN	yes	Webber 1999
PS30	myofibroblast	human	HPV16 E6/E7	SMA, VIM	yes	Price 2000
S2.13	fibroblast	human	ts-Tag	VIM	no	Daly-Burns 2007
PM151T	smooth muscle	human	hTERT	SMA, calponin, HCM	yes	Kogan 2006

Abbreviations: AR, androgen receptor; SMA, smooth muscle actin; VIM, vimentin; FN, fibronectin; HCM, heavy chain myosin; Tag, T-antigen; hTERT, human telomerase catalytic subunit.

Table 2: Urogenital sinus mesenchyme cell lines

Cell line	Phenotype	Age/Species	How Immortalized	Markers	AR	Reference
U4F	(myo)fibroblast	E18 rat	spontaneous	VIM	yes	Rowley 1992
rUGM	(myo)fibroblast	E18 rat	spontaneous	VIM	yes	Zhau 1994
UGSM-2	myofibroblast	E16 mouse	INK4a-/-	SMA, VIM	yes	Shaw 2006

Abbreviations: VIM, vimentin; SMA, smooth muscle actin

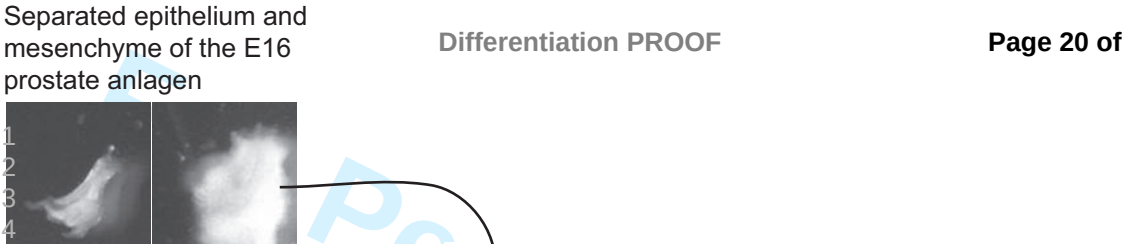

Figure 1. Isolation of UGSM-2 cells. The urogenital sinus of an E16 male INK4a-/- transgenic mouse embryo was subjected to collagenase digestion to separate epithelial (UGS-E) and mesenchymal (UGS-M) layers. UGS-M was placed into a culture dish and allowed to proliferate to confluence. Polyclonal UGSM cells were ring cloned to develop

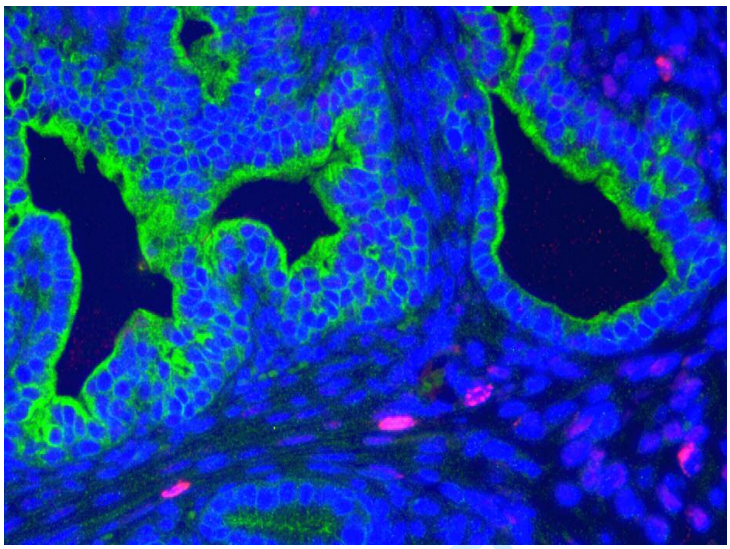

Figure 2. UGSM-2 cell fate in development of the UGS. UGSM-2 cells labeled with BrdU were seeded with dissected UGS tissue and allowed to develop under the renal capsule of male nude mice. Immunohistochemistry shows UGSM-2 cells (red) in the periductal regions of mature prostate. Epithelial cells are marked with pan-cytokeratin (green).

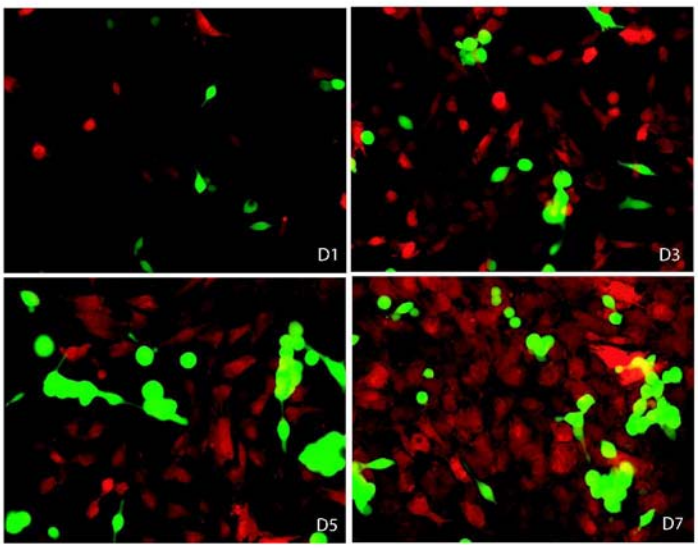

Figure 3. Labeling of cells enable tracking of cell proliferation, morphology and motility in co-cultures. UGSM-2 cells are stably labeled with monomeric RFP and LNCaP cells are labeled with GFP. This shows proliferation of each cell type over 7 days in culture. Days 1, 3, 5 and 7 are shown.

215x279mm (600 x 600 DPI)

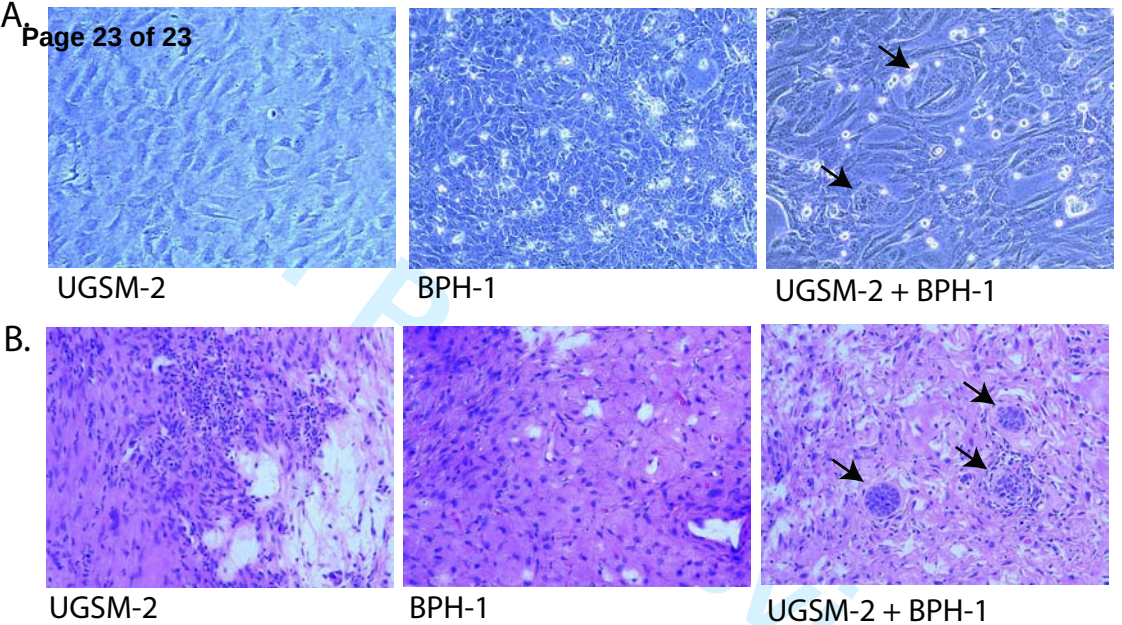

Figure 3. Interaction of UGSM-2 and human BPH-1 epithelial cells. (A) UGSM-2 cells and/or BPH-1 cells grown on rat tail collagen. Co-cultures show small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells (arrows). (B) UGSM-2 and/or BPH-1 cells renal capsule grafts after 4 weeks. Grafts of UGSM-2 cells alone yields only stromal tissue. BPH-1 cells grafted alone do not produce identifiable viable grafts. Co-grafting of UGSM-2 and BPH-1 cells results in small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells.